Spontaneous and Induced Herpesvirus Genome Expression in Marek's Disease Tumor Cell Lines

B. W. CALNEK,* W. R. SHEK AND K. A. SCHAT

Department of Avian and Aquatic Animal Medicine, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Received 6 April 1981/Accepted 7 July 1981

We incubated 31 newly established Marek's disease tumor cell lines at 41°C for 48 h after subculturing and then examined them to determine the spontaneous rates of expression of viral internal antigen(s), viral membrane antigen(s), and virus isolation. All but two of the lines were isolated from tumors induced by clone-purified Marek's disease virus strains JM-10, GA-5, RB-1B, and BC-1A in nine different genetic strains of chickens with defined histocompatibility antigens. The line-to-line variations in the rates of spontaneous expression for the antigens or virus rescue were great, but the levels of expression were very low in most cases. The median rates of expression for viral internal antigen, viral membrane antigen, and virus isolation were 32, 8, and 2 positive cells per 10⁵ cells, respectively (ranges, 0 to 20,280, 0 to 22,990, and 0 to 220 positive cells per 10⁵ cells, respectively). The ratio of viral internal antigen expression to virus isolation was extremely variable and often high, whereas the ratio of viral internal antigen to viral membrane antigen expression was more consistent and generally low. The virus strain which induced the cell line influenced the level of virus genome expression, but the cell genotype did not. Cell lines transformed by JM-10 virus, which exhibited low oncogenicity, had significantly ($P < 0.01$) higher rates of expression than cell lines transformed by GA-5 and RB-1B viruses, which exhibited high oncogenicity. Treatment with iododeoxyuridine or incubation at 37°C induced increased rates of expression in most lines but not in all lines. The degree of enhanced expression was inversely proportional to the rate of spontaneous expression.

Marek's disease (MD) is a herpesvirus-induced, malignant lymphoma of chickens (6). The transformed elements are thymus-derived (T) lymphocytes, although tumors generally contain a variety of nontransformed cells as well, including bursa-derived (B) and T lymphocytes, plasma cells, and macrophages. Because the cell populations in tumors are heterogeneous, the establishment of continuous lines of MD tumor cells in vitro has been very important since it has provided the opportunity to characterize transformed cells and to study various aspects of virus-cell interactions.

Several features of lymphoblastoid MD tumor cell lines have been described previously (1, 6, 11, 14, 17, 22-24, 32). In addition to T-cell markers, an MD tumor-associated surface antigen is uniformly present. A small proportion of cultured cells express a viral internal antigen(s) (VIA) or a viral membrane antigen(s) (VMA) or yield viruses after cocultivation with permissive cell cultures. Multiple copies of the MD virus (MDV) genome have been found in cells of the lines which have been examined (21, 30). The

complete genome appears to be present in most cases. Exceptions may be the cell lines that have been established from Sevoian's transplantable lymphoma (strain JM-V). It has been speculated that part of the genome is missing in these cell lines since all efforts to rescue virus have failed (18, 22).

The variable but generally low level (often only 1 to 2%) of virus genome expression observed in MDV-transformed cell lines is similar to the situation observed with other herpesvirus-transformed lines (8, 15). In each case, it has been shown that some cells which do not otherwise express viral antigens may do so after treatment with iododeoxyuridine (IUdR) or bromodeoxyuridine (7, 8, 10, 12, 20, 25, 28). Furthermore, IUdR treatment of MD cell lines caused increased virus isolation rates without the production of viral particles, suggesting that derepression of the viral genome could facilitate genome transfer, as well as increase production of viral antigens (20). Viral genome expression also seems to be enhanced by cultivating cells at temperatures lower than 41°C (2), and some

evidence has suggested that long-term cultivation of cell lines may reduce the percentage of cells that express genome functions (2, 8, 11, 24).

With very few exceptions, all of the MD lines that have been studied to date were unrelated. Only a very few were induced by clone-purified virus or were derived from birds of known genotype. We described an improved method for establishing MD cell lines previously (6) and have since made further technical improvements, which have resulted in the establishment of 31 new cell lines. Because many of these lines share histocompatibility antigens or were induced by the same clone of MDV or both, we were able to determine whether the virus-cell interactions which dictated the extent of virus genome expression were correlated with either cell genotype or virus strain. In addition to quantitating VIA and VMA expression and virus isolation rates, we studied the effects of IUdR and incubation at a low temperature (37°C) on these rates.

MATERIALS AND METHODS

Experimental birds. The donors of blood or tumors from which the MD cell lines were developed represented a total of 10 genetic strains of chickens. The designations and histocompatibility genotypes of these animals are listed in Table 1. GB-1 (31) donors were from a flock established at Cornell University

from hatching eggs kindly supplied by Louis Schierman, New York Medical College, Valhalla. Similarly, a flock of UDC-003 inbred chickens (9) was started from eggs generously provided by Hans Abplanalp, University of California, Davis. The UDC-003 strain is fixed for the major and minor histocompatibility antigens. The P-2 and N-2 flocks were sublines of P-lines and N-lines, which have been described previously (26). These sublines were selected for the uniformity of the B- and C-locus alleles, which determine the major histocompatibility antigen and one minor histocompatibility antigen. The S strain has been described previously (13). Some donors were obtained as 1-day-old chicks which were hatched elsewhere and were infected with MDV at Cornell University as part of collaborative projects; UDC-253, -254, -335, and -336 were provided by Hans Abplanalp and were congenic with UDC-003 (different alleles only at the B-locus), and the line 7 bird was part of a project conducted with Douglas Gilmour, New York University Medical Center, New York.

All donor flocks were free of common avian pathogens, including lymphoid leukosis virus. Experimental birds were infected with MDV when they 1 day to 3 weeks old, and they were held in isolation units until MD developed. Tumors and heparinized whole blood were collected aseptically from affected birds.

Virus strains. Cloned MDV isolates JM-10 and GA-5 have been described previously (3). Isolate BC-1, which was rescued from MSB-1 cells (19), was used uncloned and after clone purification (BC-1A). RB-1B virus was recently isolated and cloned (Schat et al.,

TABLE 1. *Newly established MD lymphoblastoid cell lines^a*

Genetic strain	Genotype	Cell lines induced by virus strain:			
		BC-1 ^b	JM-10	GA-5 ^c	RB-1B ^d
S strain	Unknown	CU-16(38), CU-18(8) ^e			
GB-1	$B^{13}B^{13}$		CU-13(13)	CU-19(13)	
P-2	$B^{19}B^{19}, C^2C^2$	CU-24(8)	CU-27(8), CU-32(4), CU-33(5), CU-35(10)	CU-12(14), CU-14(19), CU-15(35), CU-17(28)	CU-39(11), CU-42(11), CU-40(12), CU-41(11), CU-43(6)
N-2	$B^{21}B^{21}, C^2C^2$			CU-21(18), CU-22(9), CU-23(9)	
L-7	B^2B^2		CU-20(12)		
UCD-003	$B^{17}B^{17}, A^4A^4,$ C^2C^2			CU-25(14), CU-29(14), CU-38(17), CU-36(15), CU-37(12)	
UCD-335	$B^{19}B^{19f}$		CU-28(17)		
UCD-253	B^6B^{6f}			CU-30(25)	
UCD-254	$B^{15}B^{15f}$			CU-31(14)	
UCD-336	B^9B^{17f}			CU-26(24)	

^a Cell lines CU-19, CU-25, CU-26, CU-28, CU-30, CU-40, and CU-41 were from male donors; for lines CU-12, CU-13, CU-15, CU-17, CU-32, and CU-43 the sex of the donors was unknown; all other cell lines were from female donors.

^b CU-16 and CU-18 were induced by uncloned BC-1 virus; CU-24 was induced by a clone of BC-1 (BC-1A).

^c CU-21, CU-22, and CU-24 were derived from lymphomas in different organs of the same animal; CU-29 and CU-38 were derived from lymphomas in different organs of a second bird; and CU-36 and CU-37 came from lymphomas in different organs of a third bird.

^d CU-39 and CU-42 were derived from lymphomas in different organs of the same bird, and CU-40 and CU-41 came from lymphomas in different organs in another bird.

^e The numbers in parentheses are the passage levels used in this study.

^f Other alleles are congenic with UCD-003.

unpublished data). In comparative studies, strain JM-10 was judged to be moderately oncogenic, GA-5 was judged to be highly oncogenic, and RB-1B was judged to be very highly oncogenic (K. A. Schat, B. W. Calnek, J. Fabricant, and H. Abplanalp, *Poultry Sci.*, in press). The relative oncogenicity of BC-1 was not established. All virus inocula consisted of infected chicken kidney cultures.

Cell lines. We developed 31 new lymphoblastoid cell lines. The line designations, the inducing viruses, and the cell histocompatibility genotypes are given in Table 1. Most lines were derived from individual primary lymphomas in muscle or viscera. Exceptions were lines CU-12 and CU-14, which were from peripheral blood lymphocytes of leukemic birds; CU-13, which was initiated from bird passage 3 of a transplantable lymphoma; and CU-32, which was from a pool of three spleens (only one was grossly enlarged). One donor bird yielded three cell lines (from lymphomas in different organs), and four others yielded two cell lines each (Table 1).

The new lines were initiated and grown at 41°C by previously described methods (6), except for the following two significant modifications: (i) equal parts of Leibovitz L-15 and McCoy 5A media (GIBCO Laboratories, Grand Island, N.Y.) were mixed and substituted for RPMI 1640 medium in Hahn medium (designated LM-Hahn medium) and (ii) cells were grown in a humidified atmosphere containing approximately 5% CO₂ in air rather than in tightly sealed vessels. Initial passages were generally made in 1-ml cultures maintained in plastic Multiwell plates (catalog no. 3008; Falcon Plastics, Oxnard, Calif.). Later passages were made in plastic flasks (Falcon Plastics) with loose caps. A few lines were started in another modification of Hahn medium (McCoy 5A medium as the base and 5% chicken serum as the only serum supplement), but they were changed to LM-Hahn medium for these studies. The success rate for establishing new cell lines from primary lymphomas was about 50% when these methods were used. Subcultures were made every 2 to 3 days, and characterization studies were performed after 4 to 53 passages.

Characterization studies. Each cell line which was characterized for virus genome expression was examined by a standard protocol. Four cultures were prepared in parallel from each line; two were seeded at a concentration of 2.5×10^5 cells per ml in LM-Hahn medium, and two were seeded at 5.0×10^5 cells per ml in Hahn medium containing 25 µg of IUdR per ml. One culture of each type was incubated at 41°C, and the other was incubated at 37°C.

After 48 h of incubation, cell growth and viability were determined by adding trypan blue dye (final concentration 0.05%) and counting the stained (dead) and unstained (live) cells with a hemacytometer. Each culture was then examined for the following three types of viral genome expression: virus isolation, VIA, and VMA.

Virus isolations were performed by cocultivating 1×10^6 or 2×10^5 cells on primary chicken kidney monolayers in 35-mm plastic petri dishes. The number of plaque-forming units was determined 7 days later and recorded in terms of plaque-forming units per 10^5 viable cells inoculated.

The two viral antigens were detected by immuno-

fluorescence, using a fluorescein isothiocyanate-conjugated MDV antibody preparation described elsewhere (5). This preparation stained both VMA and VIA which were associated with MDV-infected chicken kidney cells. For these tests, 2×10^6 cells were washed once with modified phosphate-buffered saline, which consisted of 2% bovine serum albumin and 0.1% sodium azide in phosphate-buffered saline (pH 7.3). These cells were then suspended in 0.2 ml of MDV conjugate that was diluted in equal parts of tris(hydroxymethyl)aminomethane-buffered saline (pH 8.7) and modified phosphate-buffered saline. After 10 min at room temperature, the cells were again washed and then suspended in 0.1 ml of modified phosphate-buffered saline; 10-µl drop smears containing 200,000 cells were made on duplicate glass slides and air dried. The smears on one slide were fixed for 3 to 4 min in methyl alcohol, rinsed quickly three times in tris(hydroxymethyl)aminomethane-buffered saline (pH 7.3), and covered with buffered glycerine and a cover slip. These preparations were examined for VMA. The smears on the second slide were fixed in acetone for 10 min at room temperature and then stained for 30 min at 37°C with MDV conjugate diluted in tris(hydroxymethyl)aminomethane-buffered saline (pH 8.7). Stained smears were rinsed for 10 min in tris(hydroxymethyl)aminomethane-buffered saline (pH 7.3), cover slips were applied as described above, and the smears were examined for VIA.

Stained smears were examined and the number of positive cells was determined by using epi-illumination and a Leitz Dialux fluorescence microscope. The number of positive cells was estimated by examining a known proportion of the smear when very many cells were positive. The incidence of VMA- or VIA-positive cells was recorded as the number of positive cells per 10^5 viable cells.

In several trials, some of the cell lines were assayed at more than one passage level. The results from the lowest passage tested (never higher than passage 35) were used for comparative purposes.

Statistical analyses. Data were compared for statistical significance by using the chi-square test, the Wilcoxon signed rank test, the Wilcoxon two-sample rank test, and one-way analysis of variance (29).

RESULTS

Spontaneous genome expression. In tests on the lowest passages of the 31 lines, 26 lines (83.9%) yielded virus upon cocultivation with chicken kidney cells, 28 lines (90.3%) contained VIA-positive cells, and 23 lines (74.2%) contained VMA-positive cells. One of the five virus-negative lines also lacked VIA and VMA, whereas four contained small numbers of cells with VIA or VMA or both. All of the five virus-negative lines yielded viruses in assays of other passages or after IUdR induction (see below), and therefore they could not be classified as true nonproducers.

There was a great deal of variation from line to line with respect to all three types of genome expression (Table 2), although the median val-

TABLE 2. *Virus genome expression in MD lymphoblastoid cell lines grouped according to genetic strain of the donor bird*

Genetic strain	No. of lines	Median no. of positive cells per 10 ⁵ cells		
		VIA expression	VMA expression	Virus isolation
P-2	14	104 (1–18,367) ^a	33 (<1–22,992)	2 (0–172)
N-2	3	27 (3–136)	0 (0–114)	8 (2–66)
UCD-003	5	37 (6–20,283)	2 (0–2,248)	2 (0–3)
UCD congenic with UCD-003	4	4 (0–2,847)	0 (0–76)	<1 (0–52)
S strain	2	3 (0–67)	2 (1–4)	7 (1–13)
GB-1	2	314 (101–477)	227 (0–454)	44 (22–66)
L-7	1	3,772	2,338	219
All strains	31	32 (0–20,283)	8 (0–22,992)	2 (0–219)

^a Numbers in parentheses are ranges.

ues for VIA expression, VMA expression, and virus isolation in terms of number of positive cells per 10⁵ were low. With individual lines there were few exceptions to the general rule that the rate of VIA expression exceeded both the rate of VMA expression and the rate of virus isolation. The ratio of VIA expression rate to virus isolation rate was less than 1.0 for only three virus-positive lines, and all of these yielded less than 0.15 plaque-forming unit per 10⁵ cells. In all other lines the ratio was more than 2.2 (median, 19.2); in 74% the ratio was more than 5.0. The highest ratio of VIA expression rate to virus isolation rate was more than 10,000. VMA expression rates tended to be only slightly lower than the rates of VIA expression. The median ratio of VIA expression rate to VMA expression rate was 3.1, and only 39% of the lines had ratios of more than 5.0. Six lines were VIA positive but VMA negative, whereas only a single line was the reverse.

We also examined the data to determine whether the rate of genome expression correlated with either the cell genotype or the infecting virus. Table 2 shows the median values and ranges for the rates of VIA expression, VMA expression, and virus isolation in all 31 cell lines grouped by genetic strain of the donor; there was no apparent pattern to suggest that expression was correlated with genetic strain, and there was considerable variation within groups.

In contrast, the virus isolate had a significant effect on genome expression. Table 3 shows that the JM-10-induced lines had markedly higher median rates of expression of VIA, VMA, and virus isolation than the lines induced by any of the other viruses. When Wilcoxon two-sample

rank tests were used, both VIA expression and virus isolation rates were significantly greater ($P < 0.01$) in JM-10-induced lines than in either GA-5-induced lines or RB-1B-induced lines. In contrast, the differences between GA-5-induced lines and RB-1B-induced lines were not significant ($P > 0.05$). To illustrate this point further, the data from the two larger groups of JM-10-induced lines and GA-5-induced lines are presented in Fig. 1. Only 2 of the 16 GA-5-induced lines had rates of VIA expression which exceeded the lowest rate in the JM-10-induced lines. These lines were CU-36 and CU-37, which were from the same donor bird. The same two lines had the highest and fourth highest rates of VMA expression.

With the same cell line, variability from test to test was relatively small when the cells were passaged fewer than 35 times. A total of 12 lines were assayed two or three times with cultures separated by 1 to 26 passages. Between-test variation in the rate of VIA expression was less than 3-fold with one-half of the lines and exceeded 15-fold in only one case. In contrast, the between-line variation was significantly greater ($P < 0.01$, analysis of variance) and exceeded 10,000-fold in some instances. Similar patterns were observed with the rates of VMA expression and virus isolation. One line was also tested after 53 passages. In that case, there was a substantial (100- to 1,000-fold) decrease in the proportion of cells that expressed antigens or yielded virus compared with assays of passage 19 cells. The same line assayed at passage 35 did not show unusual decreases in the levels of expression.

Five donor birds were the sources of more than one cell line each. Pairs of lines derived from tumors in different organs were obtained from four birds, and one bird provided three separate lines (Table 1). It appeared that there was a host effect on genome expression. Among these 11 lines, the two highest rates of VIA expression (CU-36 and CU-37) and the two lowest rates of VIA expression (CU-39 and CU-42) were from pairs of lines, and the highest virus isolation rates were from the three lines derived from a single bird (CU-21, CU-22, and CU-23). The five RB-1B-induced lines comprised two pairs of lines and a single line. The pairs had very similar genome expression rates. For example, the VIA expression rates for lines CU-39 and CU-42 were 2 and 1 positive cells per 10⁵ cells, respectively, and for lines CU-40 and CU-41, the rates were 28 and 2 positive cells per 10⁵ cells, respectively. In contrast, the VIA expression rate for line CU-43 (the single line) was very different from both pairs (889 positive cells per 10⁵ cells). The same was true for VMA expression and virus isolation rates.

TABLE 3. Virus genome expression in MD lymphoblastoid cell lines grouped according to inducing virus

Virus isolate	No. of lines	Median no. of positive cells per 10^5 cells ^a		
		VIA expression	VMA expression	Virus isolation
JM-10	7	3,212 (477-18,368) ^b	1,417 (76-5,412)	52 (1-219) ^b
GA-5	16	23 (0-20,283) ^c	1 (0-2,248)	2 (0-66) ^c
RB-1B	5	2 (1-889) ^c	3 (1-125)	1 (0-3) ^c
BC-1	3	67 (0-154)	4 (1-49)	13 (1-15)

^a The numbers in parentheses are ranges.

^{b,c} Data followed by the same superscript letter are not significantly different ($P > 0.05$, Wilcoxon two-sample rank test). Different superscript letters indicate significant differences ($P < 0.01$). Data without superscript letters were not analyzed.

FIG. 1. Virus genome expression in MD cell lines induced by JM-10 virus (○) or GA-5 virus (●). Each symbol represents the rate of expression for a single cell line. Horizontal bars indicate medians. VI, Virus isolation.

Induction of virus genome expression by IUdR or incubation at 37°C. The addition of 25 μ g of IUdR per ml to the culture medium had a marked effect on some lines and little or no effect on others. Incubation at 37°C rather than 41°C also enhanced genome expression, but to a much lower extent than IUdR treatment. Using the rates of spontaneous genome expression as the basis for comparison, we determined the relative levels of expression after IUdR treatment and after incubation at the low temperature. The median relative levels of VIA expression and virus isolation were 14.3 and 12.1, respectively, for IUdR-treated cultures (ranges, 0.5 to 1,365 and 0.1 to 359.0, respectively); for cultures incubated at 37°C, these values were 4.0

and 2.1, respectively (ranges, 0.1 to 732 and 0.1 to 47.4, respectively). As in spontaneous expression rates, there was considerable variation from line to line in the extent of enhanced expression. Interestingly, this variation was related to the level of spontaneous expression (Table 4). There was a significant inverse relationship between the spontaneous expression level and the number of lines showing a fivefold or greater increase in the rate of expression for both VIA and virus isolation ($P < 0.05$, chi-square test) in treated cultures. The inverse relationship between the $\log_{10}$ level of spontaneous expression and the $\log_{10}$ ratio of IUdR-induced expression to spontaneous expression of VIA was significant ($P < 0.01$), as determined by one-way analysis of variance. This inverse relationship was true for each of the virus groups.

We also examined the effect of virus strain on enhancement of genome expression by IUdR or incubation at 37°C (Table 5). The signed rank test was used to determine whether enhancement was significant; it was significant for VIA expression and virus isolation in all GA-5-induced and RB-1B-induced cultures but only for VIA expression in JM-10-induced cultures. There were too few BC-1-induced cultures for analysis. When the relative levels of enhancement of VIA expression in lines induced by different viruses were compared by using the two-sample rank test, the differences were not significant, even though a significant effect of virus on spontaneous expression was shown (see above).

IUdR inhibited cell growth uniformly, but the toxic effects of this compound were variable. Some IUdR-treated cultures were unaffected, whereas others were affected markedly, having viability levels less than 10% the viability levels of the untreated control cultures. Of the 20 lines in which a 50% or greater reduction in viability accompanied IUdR treatment, 16 (80%) showed a fivefold or greater increase in VIA expression. Only 4 of 11 (36%) lines without a similar decrease in viability showed enhanced VIA expression. This difference was significant ($P < 0.025$,

TABLE 4. Correlations between rate of spontaneous expression of virus genome and the ability of IuDR or incubation at 37°C to enhance genome expression in MD lymphoblastoid cell lines

Type of expression	Level of spontaneous expression (no. of positive cells per 10 ⁵ cells)	IuDR treatment				Incubation at 37°C			
		No. of lines with a relative expression rate of ^a :			No. of lines with a relative expression rate of >5/total no.	No. of lines with a relative expression rate of ^a :			No. of lines with a relative expression rate of >5/total no.
		<5	5-100	>100		<5	5-100	>100	
VIA	<10	1	5	4	9/10 ^b	3	6	1	7/10 ^b
	10-1,000	4	8	2	10/14 ^b	8	6	0	6/14 ^b
	>1,000	6	1	0	1/7 ^c	7	0	0	0/7 ^c
Virus isolation	<10	2	4	6	10/12 ^b	4	8	0	8/12 ^b
	10-1,000	7	9	0	9/16 ^{b,c}	10	6	0	6/16 ^{b,c}
	>1,000	3	0	0	0/3 ^c	3	0	0	0/3 ^c

^a Relative expression rate = rate in treated culture/spontaneous rate in untreated culture.

^{b,c} Values followed by the same superscript letter were not significantly different ($P > 0.05$), as determined by the chi-square test.

TABLE 5. Effects of IuDR and incubation at 37°C on virus genome expression in MD lymphoblastoid cell lines

Virus isolate	No. of lines	Median relative levels of genome expression after ^a			
		IuDR treatment		Incubation at 37°C	
		VIA	Virus isolation	VIA	Virus isolation
JM-10	7	2.7 (1.4-13.2) ^b	0.8 (0.1-12.1)	1.6 (1.2-5.0) ^b	1.1 (0.2-4.3)
GA-5	16	10.6 (0.5-168.1) ^b	26.6 (0.7-359.0) ^b	4.8 (0.1-12.7) ^c	3.5 (0.3-25.8) ^c
RB-1B	5	1,002.0 (25.9-1,365.0) ^c	81.0 (39.5-279.8) ^c	5.3 (4.0-73.2) ^c	7.0 (6.0-47.4) ^c
BC-1	3	53.3 (2.2-3,375.0)	2.4 (0.5-171.4)	1.8 (0.1-21.7)	1.8 (0.1-21.7)

^a Relative levels were calculated as follows: number of positive cells per 10⁵ cells in treated culture/number of positive cells per 10⁵ cells in untreated culture of same line. The numbers in parentheses are ranges. The statistical significance of the difference between untreated and treated cultures was determined by the signed rank test; BC-1 cultures were not analyzed because there were too few samples.

^b Difference significant at $P < 0.01$.

^c Difference significant at $P < 0.05$.

chi-square test). No such correlation was observed with virus isolation rates. Incubation at 37°C had no adverse effect on viability, although growth rates were generally lower than at 41°C.

DISCUSSION

Our data confirm and extend the findings of others and add significant new information to help improve our understanding of cell-oncogenic herpesvirus interactions. This is the first report of comparative data on virus genome expression in a large number of cell lines in which a standard protocol for assays was used. More important, it is the first time that lines with single variables, such as inducing virus or cell genotype, could be compared. Our experimental protocol minimized artifacts. All cell lines were examined after a uniform subculture period. This was important because, as Nazerian and Witter (23) have pointed out, the percentage of antigen-positive cells increases with incubation time after subculturing. Conversely, several workers (2, 11, 25) have suggested that long-term cultivation diminishes the percentage of

antigen-positive cells. This effect was obviated by studying cultures at relatively low passage levels. The validity of comparing virus genome expression rates in different cell lines was corroborated by performing multiple assays on some of the cell lines. This study showed significantly greater variation in virus genome expression between assays of different cell lines than between assays of the same cell line.

Although chicken strains with the high degree of inbreeding found in some strains of mice are not available, many strains which share major histocompatibility antigens or major and minor histocompatibility antigens have been developed, and several are considered to be inbred. This, coupled with the availability of clone-purified virus strains differing in oncogenic potential and the improved methods for establishing continuous cell lines, makes the MD model unique. Such a study is virtually impossible with Epstein-Barr virus-infected cell lines derived from human patients or cell lines from other animal systems (e.g., simian herpesvirus-induced lymphomas).

Many of the observations in this study were not surprising. Variability among cell lines in the expression of VIA or VMA or in virus rescue rates was anticipated from previous studies (6, 11, 14, 17). Also, there were reports which showed that VIA and VMA expression rates were similar to one another or were somewhat higher in the case of VIA (1, 14, 17). Few investigators have quantitated virus isolations, but those who have reported data which suggest that antigen expression occurs at higher rates than virus rescue (6, 25). Our study provides more definitive data on this subject. It was clear that virus isolation was the least frequent manifestation of genome activity. The median virus isolation rate was 4-fold lower than the rate of VMA expression and 16-fold lower than the rate of VIA expression. Indeed, virus was never rescued from more than 0.2% of the cells, whereas the range for VMA expression and the range for VIA expression both extended from 0 to more than 20% of the cells. The ratio of VIA expression rate to virus isolation rate varied from less than 1 to more than 10,000, and thus it was not possible to predict the level of one based upon the level of the other. On the other hand, VIA expression and VMA expression rates were frequently similar in a given line. A low rate of expression probably does not mean that only a few cells carry a genome which can be expressed since 10 clones of cells from MSB-1-induced tumor cell lines all had low levels of VIA and VMA expression, which were similar to the levels of expression in the parent line (1).

By far the most exciting finding in this study was the observation that an infecting virus isolate can influence the rate of genome expression. Three of the four viruses used to induce the tumors from which our cell lines were derived have been compared for relative oncogenicity in several genetic strains of chickens which have different susceptibilities to the disease (Schat et al., unpublished data). The rates of genome expression in the various cell lines generally were inversely proportional to the oncogenicities of the isolates. Lines induced by the very highly oncogenic RB-1B virus had the lowest median values for both VIA expression and virus isolation, whereas lines induced with the much less oncogenic JM-10 virus had the highest. The GA-5 virus is also highly oncogenic, but somewhat less so than the RB-1B virus; VIA expression and virus isolation rates from GA-5-induced lines were also intermediate, but they were closer to the rates of the RB-1B-induced lines than to the rates of the JM-10 lines. This virus-associated characteristic cannot be invariable since the highest rate of VIA expression in all 31 lines was observed in a GA-5-infected culture,

and there was considerable variation even among lines infected with the same virus; rather, it appears that the probability of the virus genome in a given cell being turned-on to translate viral structural protein or to transmit infection to permissive monolayers is increased with certain viruses.

Cell genotype appears to have no effect. There were only five RB-1B-induced lines, and all were from the same chicken strain (P-2); however, this was not true of either JM-10-induced lines or GA-5-induced lines. Each of these groups was comprised of lines representing four or five genetic strains, and the differences in viral genome expression between genetic strains were minimal and not significant. On the other hand, we did observe that cell lines from tumors from a single donor tended to be more like one another than like lines from other donors in terms of genome expression. This supports the conclusion of Lee et al. (16) that a stable virus-host interaction exists in a given bird. These authors compared tumors induced by GA and JM viruses and found that the mean number of genome equivalents per cell varied from tumor to tumor when different birds were the donors but did not vary among different tumors from the same bird.

The reason for the uniformly higher spontaneous genome expression in the JM-10-induced lines is not clear, but presumably it relates to some aspect of the virus-cell relationship in which JM-10 interacts in a manner slightly different from GA-5 or RB-1B. It should be interesting to determine whether high-expression lines carry more virus genome equivalents per cell than low-expression lines and whether the extent of integration of the viral genome with the cellular deoxyribonucleic acid correlates with the rate of turn-on.

In any event, the relationship between virus strain and genome expression in the cell lines is potentially significant *in vivo*, since it could relate to oncogenicity. Two possible relationships can be considered. The first is that virus genomes that are more likely to turn-on in transformed or potentially transformable T lymphocytes might do so to the extent that cytolytic infection ensues and the cell does not survive. Thus, more stable latency associated with cells transformed by more oncogenic strains of MDV might enhance the probability of tumor formation. However, our turn-on rates *in vitro* were generally low and did not prevent cell proliferation and the establishment of cell lines. Moreover, we gathered no data comparing the rate of cell division with the turn-on rate.

A second possibility is that the high percentage of VMA-bearing cells in *in vitro* cell lines induced by JM-10 MDV could be associated

with a high susceptibility of transformed cells in vivo to immune attack. Antiviral immune responses would be irrelevant if all of the cells that expressed VMA inevitably underwent virus-mediated cytolysis. Conversely, it is plausible that qualitative and quantitative differences in VMA expression, as well as expression of non-serologically detected surface antigen (25, 27), could occur, allowing cells to multiply and translate viral proteins in the absence of virus-mediated cytopathic effects. Currently, there are no data showing qualitative differences in VMA expressed on cells transformed by various strains of MDV. Furthermore, although JM-10-induced cell lines had higher rates of expression of VMA than GA-5- and RB-1B-induced cell lines, the rate of expression was generally low and therefore might not explain the different oncogenic potentials of the various MDV strains.

The enhancement of virus genome expression by IUdR was expected, based on other reports. What was not expected was the wide variation from line to line in the effectiveness of the treatment or the correlation between low spontaneous expression and high relative inducibility. We should be cautious in interpreting the results of these experiments. Whereas the proportional increase in the number of VIA-positive cells after IUdR treatment was greatest with the lines that displayed the lowest spontaneous expression rates, the actual numbers of induced cells per 10^5 cells were similar in lines with high and low spontaneous expression rates. Essentially, our data suggest that regardless of the virus, high-expression lines may produce antigens or yield virus at rates close to their potential, whereas lines with low expression rates do not.

The only other study with comparable data is the study of Dunn and Nazerian (7). These authors compared MSB-1 high-producer cultures with low-producer cultures and found that the latter were activated to a lower degree than the former by IUdR. However, the data of these workers indicated essentially similar proportional changes in the number of VIA-positive cells after induction. It should be remembered that in our study the differences were between cell lines rather than between different cultures of the same line. The low-producer lines of Dunn and Nazerian had been passaged more than 80 times, and the potential turn-on of cells in the culture population may have been modified.

We made no attempt to characterize the mechanism of IUdR induction or the type of antigen(s) present in normal or induced cells. This subject has been studied by others (7, 20, 25, 28). However, it seems possible that there are qualitative as well as quantitative differences

among cell lines in antigen production, either spontaneous or induced.

Collectively, the findings discussed above suggest that MD tumor cell lines which share selected characteristics can be potent tools for determining factors which are important in herpesvirus oncology.

ACKNOWLEDGMENTS

We thank R. W. Harris for excellent technical assistance and G. Troise for typing the manuscript.

This investigation was supported in part by Public Health Service grant 5 R01 CA-6709-19 from the National Cancer Institute.

LITERATURE CITED

1. Akiyama, Y., and S. Kato. 1974. Scanning electron microscopy of lymphoid cell lines and lymphocytes from thymus, bursa and blood of chickens. *Biken J.* 17:193-197.
2. Arita, K., and S. Nii. 1979. Effect of culture temperature on the production of Marek's disease virus antigens in a chicken lymphoblastoid cell line. *Biken J.* 22:31-34.
3. Calnek, B. W. 1973. Influence of age at exposure on the pathogenesis of Marek's disease. *J. Natl. Cancer Inst.* 51:929-939.
4. Calnek, B. W. 1980. Marek's disease virus and lymphoma, p. 103-143. *In* F. Rapp (ed.), *Oncogenic herpesviruses*. CRC Press, Boca Raton, Fla.
5. Calnek, B. W., J. C. Carlisle, J. Fabricant, K. K. Murthy, and K. A. Schat. 1979. Comparative pathogenesis studies with oncogenic and nononcogenic Marek's disease viruses and turkey herpesvirus. *Am. J. Vet. Res.* 40:541-548.
6. Calnek, B. W., K. K. Murthy, and K. A. Schat. 1978. Establishment of Marek's disease lymphoblastoid cell lines from transplantable versus primary lymphomas. *Int. J. Cancer* 21:100-107.
7. Dunn, K., and K. Nazerian. 1977. Induction of Marek's disease virus antigens by IUdR in a chicken lymphoblastoid cell line. *J. Gen. Virol.* 34:413-419.
8. Falk, L. A., Jr. 1980. Simian herpesviruses and their oncogenic properties, p. 145-173. *In* F. Rapp (ed.), *Oncogenic herpesviruses*. CRC Press, Boca Raton, Fla.
9. Feshing, M. F. W. 1979. Inbred strains in biomedical research. McMillan, London.
10. Gerber, P. 1972. Activation of Epstein Barr virus by 5-bromo-deoxyuridine in "virus-free" human cells. *Proc. Natl. Acad. Sci. U.S.A.* 69:83-85.
11. Hahn, E. C., L. Ramos, and A. J. Kenyon. 1977. Lymphoproliferative diseases of fowl: JM-V leukemic lymphoblasts in cell culture: brief communication. *J. Natl. Cancer Inst.* 59:267-271.
12. Hampar, B., J. G. Derge, L. M. Martos, and J. L. Walker. 1972. Synthesis of Epstein-Barr virus after activation of the viral genome in a "virus negative" human lymphoblastoid cell (Raji) made resistant to 5-bromo-deoxyuridine. *Proc. Natl. Acad. Sci. U.S.A.* 69:78-82.
13. Hutt, F. B., and R. K. Cole. 1953. The interaction of genetic and environmental influences affecting the incidence of avian leucosis. *Science* 117:695-697.
14. Kato, S., and Y. Akiyama. 1975. Lymphoid cell lines from lymphomas of Marek's disease, p. 101-108. *In* G. de-The, M. A. Epstein, and H. zur Hausen (ed.), *Oncogenesis and herpesviruses II*, part 2. International Agency for Research on Cancer, Lyon, France.
15. Klein, G. 1972. Herpesviruses and oncogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 69:1056-1064.

16. Lee, L. F., K. Nazerian, and J. A. Boezi. 1975. Marek's disease virus DNA in a chicken lymphoblastoid cell line (MSB-1) and in virus-induced tumours, p. 199-204. *In* G. de-The, M. A. Epstein, and H. zur Hausen (ed.), *Oncogenesis and herpesviruses II*, part 2. International Agency for Research on Cancer, Lyon, France.
17. Matsuda, H., K. Ikuta, H. Miyamoto, and S. Kato. 1976. Demonstration of Marek's disease tumor-associated surface antigen (MATSA) on six cell lines derived from Marek's disease lymphomas. *Biken J.* 19:119-124.
18. Munch, D., and M. Sevoian. 1980. Growth and characterization of, and immunological response of chickens to, a cell line established from JMV lymphoblastic leukemia. *Avian Dis.* 24:23-26.
19. Murthy, K. K., and B. W. Calnek. 1978. Pathogenesis of Marek's disease: early appearance of Marek's disease tumor-associated surface antigen in infected chickens. *J. Natl. Cancer Inst.* 61:849-854.
20. Nazerian, K. 1975. Induction of an early antigen of Marek's disease virus in a lymphoblastoid cell line, p. 345-350. *In* G. de-The, M. A. Epstein, and H. zur Hausen (ed.), *Oncogenesis and herpesviruses II*, part 2. International Agency for Research on Cancer, Lyon, France.
21. Nazerian, K., and L. F. Lee. 1974. Deoxyribonucleic acid of Marek's disease virus in a lymphoblastoid cell line from Marek's disease tumours. *J. Gen. Virol.* 25:317-321.
22. Nazerian, K., E. A. Stephens, J. M. Sharma, L. F. Lee, M. Gailitis, and R. L. Witter. 1977. A nonproducer T lymphoblastoid cell line from Marek's disease transplantable tumor (JMV). *Avian Dis.* 21:69-76.
23. Nazerian, K., and R. L. Witter. 1975. Properties of a chicken lymphoblastoid cell line from Marek's disease tumor. *J. Natl. Cancer Inst.* 54:453-458.
24. Powell, P. C., L. N. Payne, J. A. Frazier, and M. Rennie. 1974. T lymphoblastoid cell lines from Marek's disease lymphomas. *Nature (London)* 251:79-80.
25. Ross, L. J. N., P. C. Powell, D. J. Walker, M. Rennie, and L. N. Payne. 1977. Expression of virus-specific, thymus-specific and tumour-specific antigens in lymphoblastoid cell lines derived from Marek's disease lymphomas. *J. Gen. Virol.* 35:219-235.
26. Schat, K. A., and B. W. Calnek. 1978. In vitro cytotoxicity of spleen lymphocytes against Marek's disease tumor cells: induction by SB-1, an apparently nononcogenic Marek's disease virus, p. 301-316. *In* P. M. Biggs (ed.), *Resistance and Immunity to Marek's Disease*. Commission of the European Communities, Brussels.
27. Schat, K. A., and K. K. Murthy. 1980. In vitro cytotoxicity against Marek's disease lymphoblastoid cell lines after enzymatic removal of Marek's disease tumor-associated surface antigen. *J. Virol.* 34:130-135.
28. Silver, S., A. Tanaka, and M. Nonoyama. 1979. Transcription of the Marek's disease virus genome in a nonproductive chicken lymphoblastoid cell line. *Virology* 93:127-133.
29. Snedecor, G. W., and W. G. Cochran. 1971. *Statistical methods*, 6th ed. Iowa State University Press, Ames.
30. Tanaka, A., S. Silver, and M. Nonoyama. 1978. Biochemical evidence of the nonintegrated status of Marek's disease virus DNA in virus-transformed lymphoblastoid cells of chickens. *Virology* 88:19-24.
31. Theis, G. A., L. W. Schierman, and R. A. McBride. 1974. Transplantation of a Marek's disease lymphoma in syngeneic chickens. *J. Immunol.* 113:1710-1715.
32. Yamaguchi, S., H. Kawamura, H. Matsuda, and S. Kato. 1979. Establishment of Marek's disease lymphoblastoid cell lines from chickens with B^AB^K of B blood groups. *Biken J.* 22:35-40.